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Enhanced efficacy of an attenuated *Flavobacterium psychrophilum* strain cultured under iron-limited conditions

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ABSTRACT

An attenuated strain of *Flavobacterium psychrophilum* (CSF259-93B.17) has shown potential as a vaccine for prevention of bacterial coldwater disease (BCWD) in rainbow trout, *Oncorhynchus mykiss* (Walbaum). Because BCWD outbreaks can result in high mortality in other salmonid species, specifically coho salmon, *Oncorhynchus kisutch* (Walbaum), the live-attenuated strain was tested as a vaccine in this species. Additionally, we hypothesized that culture of the vaccine strain under iron-limited conditions would lead to improved protection against BCWD. To test this hypothesis, coho salmon were either injection or immersion immunized with CSF259-93B.17 cultured in iron-replete or iron-limited medium. Resultant antibody titers were low and not significantly different between the two treatments regardless of vaccine delivery method ($P > 0.05$). Following injection challenge with a virulent *F. psychrophilum* strain, mortality for injection vaccinated fish was significantly reduced compared to the control but did not differ by treatment ($P > 0.05$). Relative percent survival (RPS) was high in both treatments (90% in iron-replete, 98% in iron-limited medium). Fish immunized by immersion with CSF259-93B.17 grown in iron-replete medium exhibited lower mortality (29.3%; RPS 46%) when compared to mock immunized fish, but this was not significant. However, mortality was significantly lower in fish immunized with CSF259-93B.17 grown in iron-limited medium (14.7%; RPS 73%) when compared to mock immunized fish. The results demonstrate that the live-attenuated *F. psychrophilum* strain can confer protection to coho salmon and vaccine efficacy is enhanced by culturing the strain under iron-limited conditions.

1. Introduction

The Gram-negative bacterium *Flavobacterium psychrophilum* is the causative agent of both bacterial coldwater disease (BCWD) and rainbow trout fry syndrome (RTFS). *F. psychrophilum* has been isolated from numerous fish species but salmonids, especially coho salmon *Oncorhynchus kisutch* (Walbaum), rainbow trout *Oncorhynchus mykiss* (Walbaum), and steelhead, are most commonly affected [1–3]. Depending on size and species, BCWD and RTFS epizootics can result in mortality levels ranging from 5 to 85% of the population [4–6], and the economic impacts are extensive. Development of successful disease prevention strategies, such as implementation of an effective vaccine program, is highly desired.

Development of an injection vaccine for *F. psychrophilum* has had varying degrees of success [7–17]. While fish develop specific antibody titers against *F. psychrophilum*, relative percent survival (RPS) values are often low. The use of a strong adjuvant, such as Freund's complete adjuvant (FCA) has been shown to enhance the immune response and provide protection, but practical application of such a vaccine would be limited. A live-attenuated *F. psychrophilum* strain has been produced [18]. This strain (*F. psychrophilum* CSF259-93B.17) is non-virulent and induces protection in rainbow trout challenged with the virulent parent strain CSF259-93 [18]. However, the RPS of immunized fish in initial trials was 45% and below desired levels indicating further effort should focus on enhancing the efficacy of the CSF259-93B.17 vaccine.

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Based on results of previous studies with *F. psychrophilum* [19] as well as studies with other fish pathogens [20–23], we hypothesized that culture of the *F. psychrophilum* CSF259-93B.17 strain in medium which mimics the *in vivo* environment may enhance immunogenicity of the attenuated strain. Differences between the *in vivo* environment and the growth medium used to culture *F. psychrophilum* have been documented [24], and include nutrient concentration, osmality, and iron concentration. In the host environment, intracellular iron is bound to hemoglobin, heme, ferritin, and hemosiderin while extracellular iron is bound to lactoferrin and transferrin, which are glycoproteins with a high affinity for iron. In short, the amount of free iron available in a host for use by pathogenic bacteria is extremely limited in comparison to levels in most growth media.

The role of iron in *F. psychrophilum* growth and pathogenesis has only recently been investigated. Cells grown in iron-replete medium have differential protein regulation compared to those grown in iron-limited medium (ILM) or the *in vivo* environment. Several proteins with increased production in ILM are also increased *in vivo* [25] including an immunogenic protein recognized by a monoclonal antibody as well as other immunogenic proteins [7,25–28]. Finally, at least one *F. psychrophilum* strain (NCIMB1947) has increased membrane vesicle production when grown in ILM [29].

In the current study, we sought to evaluate the use of ILM to enhance the immunogenicity of the live-attenuated strain *F. psychrophilum* CSF259-93B.17. In addition, the efficacy of CSF259-93B.17 in coho salmon was assessed. This was accomplished by either injection or immersion immunization of juvenile coho salmon with *F. psychrophilum* CSF259-93B.17 cultured under iron-replete or iron-limited conditions. Serum samples were collected at 4, 6, and 12 weeks post-initial immunization to measure antibody titer and persistence. Six weeks post-initial immunization, fish were challenged with a virulent *F. psychrophilum* strain.

2. Materials and methods

2.1. Bacterial culture

Both the live-attenuated and virulent *F. psychrophilum* strains were cultured for 72 h at 15 °C in tryptone yeast extract salts broth (TYES; 0.4% tryptone, 0.04% yeast extract, 0.05% calcium chloride, 0.05% magnesium sulfate, pH 7.2) using previously published protocols [16,30]. The iron chelator, 2′2-dipyridyl (Sigma–Aldrich, St. Louis, MO, USA), was added to TYES broth at a final concentration of 50 μM to prepare ILM. Based on prior research in our lab, which indicated growth was higher when shaken, all CSF259-93B.17 cultures were grown on an orbital shaker at ~83 rpm. For the challenge trial, the virulent strain (CSF259-93) was grown statically [31,32] due to its tendency to agglutinate under shaking conditions.

To harvest bacteria for both the injection immunization and challenge experiments, cultures were centrifuged (4300 × *g*) for 15 min, the supernatant was poured off, and pellets were re-suspended in sterile phosphate buffered saline (PBS, pH 7.0) to the appropriate optical density value at 525 nm (OD_{525}). For immersion immunization, cultures were not treated prior to use. To estimate colony forming units (CFU) mL^{-1}, a 6 × 6 drop plate method was used in conjunction with TYES agar plates [33]. Plates were incubated at 15 °C for 96 h and colonies were counted.

2.2. Fish and rearing conditions

Eyed coho salmon eggs from Skookum Creek Hatchery (Acme, WA, USA) were transported to University of Idaho (Moscow, ID, USA) and disinfected in 100 ppm Ovadine® (Western Chemical Inc., Ferndale, WA, USA) for 10 min upon arrival. Fish were reared according to standard practices in flow-through systems using 11 °C dechlorinated municipal water. Immunized fish were maintained in 250 L tanks and fed 2% body weight d^{-1} (EWOS, British Columbia, Canada). Following the bacterial challenge, fish were held in 19 L tanks and fed 1% body weight d^{-1}. All research activities involving fish were approved by the University of Idaho Institutional Animal Care and Use Committee.

2.3. Immunization

2.3.1. Injection

Two treatments and one control group (170 fish each, mean weight 3.6 g) were included in this study. Fish were anesthetized in 100 μg mL^{-1} tricaine methanesulfonate (MS-222; Argent Chemicals, Redmond, WA, USA) and injected intraperitoneally (i.p.) with 50 μL of the appropriate treatment using a 30 gauge needle. The treatment groups received either CSF259-93B.17 cultured in iron-replete or ILM conditions, while the control group received sterile PBS as a mock immunization. At the primary immunization, cells were re-suspended to an OD_{525} of 0.5 for the CSF259-93B.17 group which corresponded to 2.1×10^8 CFU mL^{-1} (1.1×10^7 CFU $fish^{-1}$). The OD_{525} for the CSF259-93B.17 ILM group was 0.55 which corresponded to 6.7×10^5 CFU mL^{-1} (3.4×10^4 CFU $fish^{-1}$). Fish were booster immunized at 4 weeks as described above, and the determined doses were 2.0×10^7 CFU mL^{-1} (1.0×10^6 CFU $fish^{-1}$) and 1.8×10^6 CFU mL^{-1} (9.0×10^4 CFU $fish^{-1}$) for the CSF259-93B.17 and CSF259-93B.17 ILM treatments, respectively. The OD_{525} for both treatments was 0.51.

2.3.2. Immersion

Two treatments and one control group (170 fish each, mean weight 3.6 g) were included in this study. In a previous study, protection following laboratory challenge was greater in fish when adipose fins were removed prior to immersion in the live-attenuated strain [18]. As such, adipose fins were removed for all immersion vaccinated fish including the control group in this study. Fish were anesthetized in 100 μg mL^{-1} MS-222 and a sterile scalpel used to remove the adipose fin. Fish were allowed to recover for approximately 1 min prior to immunization. All groups were immersed for 1 h in 7.6 L tank water containing the treatment or control at a 1:4 (v/v) dilution with aeration. The treatment groups received either CSF259-93B.17 cultured in iron-replete or ILM conditions, while the control group received sterile TYES broth. Fish in the CSF259-93B.17 treatment were immunized using a bacterial culture with an OD_{525} value of 0.5 that corresponded to 2.1×10^8 CFU mL^{-1} (5.3×10^7 CFU mL^{-1} after dilution in water). Fish in the CSF259-93B.17 ILM treatment were immunized using a bacterial culture with an OD_{525} value equal to 0.4 that corresponded to a concentration of 2.0×10^7 CFU mL^{-1} (6.3×10^6 CFU mL^{-1} after dilution in water). Fish were booster immunized at 4 wk by immersion in 10 L of the appropriate treatment for 1 h with aeration. Volume was increased from initial immunization to booster to account for increase in fish size. We attempted to equalize the booster doses to the OD_{525} value from primary immunization. For the booster doses, the OD_{525} of the CSF259-93B.17 culture was 0.46, which corresponded to 2.1×10^7 CFU mL^{-1} (5.3×10^6 CFU mL^{-1} after dilution in water), and the OD_{525} for the CSF259-93B.17 ILM culture was 0.49, which corresponded to 3.9×10^5 CFU mL^{-1} (9.8×10^4 CFU mL^{-1} after dilution in water).

2.4. Serum collection

Serum was collected from 25 fish (five pools of five fish) prior to immunization to serve as the negative control for ELISA (see

below). At 4 and 6 wk post-primary immunization, serum was collected from all injection and immersion immunized groups by sampling 30 fish (10 pools of three fish) from each treatment. Serum was collected at 12 wk post-primary immunization by sampling from the remaining 15 fish (five pools of three fish). Samples were also collected from challenge survivors at the end of the 28 d challenge. Serum was pooled at the time of collection.

To collect serum, fish were euthanized with a lethal overdose of tricaine methanesulfonate (MS-222; Argent Chemicals, Redmond, WA, USA) and bled by severing the caudal peduncle. Blood was collected in 0.1 mL hematocrit tubes (Fisher Scientific, Pittsburgh, PA, USA). Blood was placed in 1.5 mL centrifuge tubes and allowed to clot overnight at 4 °C. The next day, samples were centrifuged (15,000 × *g*) for 5 min and sera collected. Samples were stored at −20 °C until used in the enzyme-linked immunosorbent assay (ELISA).

2.5. Bacterial challenge

At 6 wk post-primary immunization, triplicate groups of 25 fish (mean weight 7 g) from vaccinated and mock vaccinated groups were challenged according to methods of LaFrentz et al. [16]. Briefly, fish were subcutaneously injected with 25 μL of the virulent *F. psychrophilum* strain (CSF259-93) at a concentration of approximately 2.8×10^7 CFU mL^{-1} (7.1×10^5 CFU fish^{-1}; $OD_{525} = 0.358$). In each group, a subset of fish ($n = 20$) were injected with sterile PBS to serve as the mock infected control. Mortalities were monitored on a daily basis for 28 d and re-isolation of *F. psychrophilum* was attempted by sampling 20% of the daily mortality and streaking kidney, liver, and spleen samples on TYES agar and incubating plates at 15 °C for 96 h. Presumptive identification of isolates as *F. psychrophilum* was based on colony color (yellow) and morphology (convex with smooth morphology or convex with a thin spreading margin).

2.6. Enzyme-linked immunosorbent assay

An ELISA was used to determine antibody titers against *F. psychrophilum* in all groups of fish. The ELISA was done as previously described by LaFrentz et al. [16] with one modification. To reduce background, a blocking step was added prior to serum application in which 150 μL of 5% (w/v) non-fat dry milk (Bio-Rad, Carlsbad, CA, USA) + potassium phosphate buffered saline (KPBS) + 0.05% (v/v) Tween-20 (Fisher Scientific, Pittsburgh, PA, USA) was added to each well and plates incubated at room temperature for 1 h. Serum samples were diluted from 1:50 to 1:51,200 in a series of doubling dilutions in PBS containing 0.02% sodium azide. Titer was set as the reciprocal of the highest dilution that had an optical density at least two times greater than the negative control.

2.7. Statistical analyses

The cumulative percent mortality (CPM) of each treatment or control group was calculated for all experiments. The relative percent survival (RPS) was calculated for the vaccine trials using the following formula: (1 − (CPM vaccinated group/CPM unvaccinated group)) × 100 [34].

Differences in CPM and antibody titer ($\log_{10}$ transformed) were determined using a one-way ANOVA ($\alpha = 0.05$) after confirming residuals were normally distributed and variances were equal for both data sets. If the differences were significant ($P < 0.05$), a Tukey's post-hoc test was carried out to determine which groups were different. Statistical analysis was completed using GraphPad® Prism v5.03 (GraphPad, San Diego, CA, USA).

3. Results

Immunization of coho salmon with either CSF259-93B.17 or CSF259-93B.17 ILM resulted in significantly greater antibody titers against *F. psychrophilum* in both delivery methods (Table 1) as compared to mock immunized fish at 4 ($F_{5,54} = 106.2$; $P < 0.0001$), 6 ($F_{5,54} = 127.1$; $P < 0.0001$), and 12 ($F_{5,54} = 52.8$; $P < 0.0001$) wks post-immunization. The mean antibody titer (±standard error of the mean) of fish immunized by injection with CSF259-93B.17 increased from 800 ± 278 (4 wk) to 8960 ± 1568 (12 wk). For fish immunized by injection with CSF259-93B.17 ILM, the mean titer at 4 wk post-immunization was 490 ± 90 and increased to 14,720 ± 4703 at week 12. Mean antibody titers of fish immunized by immersion with both the CSF259-93B.17 and CSF259-93B.17 ILM were similar throughout the experiment and titers in this delivery method were never greater than 5400 ± 1998. Differences between titers for the regular and ILM treatments were not statistically significant for either vaccination method. In both treatments, antibody levels were significantly elevated at 12 wks post-immunization demonstrating persistence of the antibody. Antibody titers of challenge survivors also were not significantly different between treatments (data not shown).

The CPM of fish immunized by injection with either treatment was significantly lower than that of the mock-immunized fish ($F_{5,11} = 12.41$; $P = 0.0003$) (Table 2). The difference in CPM between CSF259-93B.17 and CSF259-93B.17 ILM was not significant. Relative percent survival values of the two treatments were 90% (CSF259-93B.17) and 98% (CSF259-93B.17 ILM). The CPM of fish immunized by immersion with CSF259-93B.17 ILM was significantly lower than the mock immunized control group ($F_{5,11} = 12.41$; $P = 0.0003$) while the CPM of fish immunized by immersion with CSF259-93B.17 was not (Table 2). The difference in CPM between the two treatments was not significant although it was lower in CSF259-93B.17 ILM. The RPS of the CSF259-93B.17 treatment was 46% while the RPS of the CSF259-93B.17 ILM treatment was 73% (Table 2).

Yellow-pigmented bacteria exhibiting phenotypic characteristics of *F. psychrophilum* were re-isolated from 92% (68/74) of the mortalities examined. In addition, mortalities exhibited clinical symptoms of *F. psychrophilum* infection including necrotic lesions and yellow-pigmented mats on the head.

4. Discussion

The parent strain of the live-attenuated *F. psychrophilum* strain was originally recovered from a diseased rainbow trout [32]. Initial studies with CSF259-93B.17 demonstrated protection was conferred in immunized rainbow trout challenged with the virulent strain [18]. Other salmonid species, most notably coho salmon, can also experience significant losses due to BCWD outbreaks. As host-specific genetic and serological differences have been

Table 1
Mean serum ELISA antibody titers ± standard error of the mean (SEM) at 4, 6, and 12 wk post-initial immunization.

Delivery method	Treatment	Week 4	Week 6	Week 12
Injection	Mock immunized	40 ± 7[a]	40 ± 7[a]	200 ± 55[a]
	CSF259-93B.17	800 ± 278[b]	2720 ± 697[b]	8960 ± 1568[b]
	CSF259-93B.17 ILM	490 ± 90[b]	1640 ± 374[b]	14,720 ± 4703[b]
Immersion	Mock immunized	<50[a]	<50[a]	140 ± 25[a]
	CSF259-93B.17	1480 ± 316[b]	1760 ± 261[b]	4480 ± 784[b]
	CSF259-93B.17 ILM	1680 ± 278[b]	880 ± 80[b]	5440 ± 1998[b]

Mean titer values with different superscripts indicate a significant difference ($P < 0.05$) at each sampling point within delivery method.

Table 2
Immunization dosage with corresponding optical density values, cumulative percent mortality (CPM) ± standard error of the mean (SEM), and relative percent survival (RPS) of immunized and mock-immunized coho salmon following experimental challenge with *F. psychrophilum* CSF259-93 at 6 wk post-initial immunization.

Delivery method	Treatment	Initial immunization (CFU mL^{-1}) and (OD_{525})	Booster immunization (CFU mL^{-1}) and (OD_{525})	CPM ± SEM	RPS
Injection	Mock immunized	–	–	65.3 ± 10.4^{a}	
	CSF259-93B.17	2.1×10^{8} (0.5)	2.1×10^{7} (0.51)	6.7 ± 3.5^{b}	90
	CSF259-93B.17 ILM	6.7×10^{5} (0.55)	1.8×10^{6} (0.51)	1.3 ± 1.3^{b}	98
Immersion	Mock immunized	–	–	54 ± 2^{a}	
	CSF259-93B.17	2.1×10^{8} (0.5)	2.1×10^{7} (0.46)	$29.3 \pm 10.7^{a,b}$	46
	CSF259-93B.17 ILM	2.0×10^{7} (0.4)	3.9×10^{5} (0.49)	14.7 ± 6.7^{b}	73

Cumulative percent mortality values with different subscripts indicate a significant difference ($P < 0.05$) within delivery method.

observed for *F. psychrophilum* isolates, particularly those from rainbow trout and coho salmon [35–42], it was essential to determine the efficacy of CSF259-93B.17 in coho salmon. The significantly lower CPM of both the injection and immersion immunized groups (Table 2) as compared to the mock immunized group demonstrates that CSF259-93B.17 does indeed confer protection in this species.

We hypothesized that ILM could be used to increase the immunogenicity of CSF259-93B.17 as other studies have reported higher survival in fish vaccinated with either killed bacteria [43] or outer membrane proteins [21,44] cultured under iron-limited conditions. In the injection immunized trial in the current study, mortality in both the iron-replete and ILM was low, <7%, and the difference between the two groups was not significant. This, along with the lower mortality in the injection immunized group as compared to the immersion immunized group, is likely due to the high efficiency generally observed with injection vaccination. The use of a live-attenuated strain may stimulate different immune parameters regardless of media type as compared to killed bacteria or outer membrane proteins.

Ideally, an efficacious vaccine against BCWD will be administered by immersion because outbreaks typically occur when fish are 5 g or smaller [45] making injection immunization unfeasible. In the initial report on CSF259-93B.17 [18], a pilot study wherein rainbow trout were adipose fin clipped, immersed in CSF259-93B.17, and challenged 10 weeks post-immunization resulted in a RPS of 45%, the same as that obtained in the current study for CSF259-93B.17. Differences in CPM between the control and CSF259-93B.17 were not significant in the current study. However, the CPM of the CSF259-93B.17 ILM group was significantly lower than the control. This clearly demonstrates enhanced protection using immersion vaccination when the CSF259-93.B.17 strain is cultured under iron-limited conditions.

Antibody titers, although comparable to the initial studies with CSF259-93B.17 [18], are somewhat low compared to other studies in which fish were vaccinated with killed bacteria emulsified with adjuvant [11,14–16]. LaFrentz et al. [18] hypothesized that low antibody titers may be a reflection of using antigen from the parent strain to coat the ELISA plates instead of antigen from CSF259-93B.17. When ELISAs were performed with serum from two pools of fish sampled at 4 wk from the CSF259-93B.17 group using antigen from either the parent strain or CSF259-93B.17 to coat the plates, titers were similar (data not shown). However, we did not evaluate antigen from CSF259-93.B.17 cultured under iron-limited conditions and titers may have been different had we used this strategy.

Given the disparity between RPS and antibody titers for both delivery methods, it seems likely that another aspect of the immune response such as cell-mediated immunity is stimulated by the live-attenuated strain. Low antibody titers and high protection have been noted in studies where fish were vaccinated with killed bacteria grown in ILM versus killed bacteria grown in regular media [43]. Using attenuated *Aeromonas salmonicida* and *Aeromonas hydrophila* strains results in significantly greater T-cell proliferation as compared to B-cells, a trend not observed in fish immunized with killed bacteria [46,47]. Additionally, macrophages from channel catfish, *Ictalurus punctatus*, vaccinated with a live-attenuated *Edwardsiella ictaluri* strain have significantly greater killing ability, reactive oxygen species production, and nitric oxide production as compared to those from non-vaccinated fish [48]. Although not evaluated in this study, similar processes may be occurring in fish vaccinated with CSF259-93B.17. It is also possible that other immunoglobulins, specifically IgT and IgD, are stimulated by the live-attenuated strain resulting in the increased protection. Further studies are necessary to determine the role of both the innate and adaptive immunity in protection, and the effect iron-regulated proteins have on these processes.

Optical density was standardized between treatments with the assumption that equal optical densities would translate to equivalent cell counts in all experiments. Additionally, bacteria were harvested during logarithmic growth stage which was determined by growth experiments with CSF259-93B.17 cultured in iron-replete and iron-deplete media (data not shown). Nevertheless, cell counts were lower by at least one log in ILM cultures in both experiments. One possible explanation for this difference in cell counts is that bacteria in ILM are viable but not culturable, which has been observed for *F. psychrophilum* in the absence of nutrients [24,49,50]. Another possible explanation is that growth under iron-limited conditions may result in surface changes on the cell that change the light-scattering properties of the cell which will influence optical density measurements. At least one *F. psychrophilum* strain has been shown to exhibit increased surface blebbing after growth on ILM [29]. A similar phenomenon has been reported for *Streptococcus pneumonia* and *Legionella pneumophila* cultures grown in iron-depleted media [51,52]. Despite the lower immunizing dose for CSF259-93B.17 ILM, protection was still enhanced over the strain grown in iron-replete media.

Although the exact mechanism is unknown, results of the current study indicate that use of ILM enhances the efficacy of a live-attenuated *F. psychrophilum* strain. There are six proteins produced by *F. psychrophilum* that are similarly expressed in both ILM and the *in vivo* environment, including the immunogenic gliding motility protein GldN [25]. As gliding motility and proteolytic activity have been implicated in aiding the infection process, up-regulation and increased production of this protein and others may alter the ability of CSF259-93B.17 to invade and colonize the host [26]. It is also possible that, when grown in ILM, the live-attenuated strain is more immunogenic due to increased protein expression. This is likely a result of up-regulation of genes encoding for iron-regulated outer membrane proteins (IROMPS), although that has not been confirmed. These proteins are known to increase virulence in both human and fish pathogens [21,43,53].

Increased expression of iron acquisition mechanisms in *F. psychrophilum* when grown under iron-limited conditions may

also be a factor. Pathogenic bacteria use several different mechanisms for iron acquisition including siderophore production, binding iron to the surface of the bacteria, conversion by ferroxidase, and production of hemolysins [54]. These mechanisms, particularly hemolysin synthesis, may be increased in Gram-negative bacteria when iron is limiting [55]. *F. psychrophilum* encodes one protein with a 53% similarity at the amino acid level to a *Vibrio anguillarium* hemolysin, and several TonB outer membrane iron receptors and proteins similar to ferrous transport proteins FeoA and FeoB [56]. Additionally, production of siderophore-like molecules has been observed for some strains grown in ILM [57,58].

In conclusion, results from this study show that the live-attenuated *F. psychrophilum* CSF259-93B.17 strain can be used to reduce mortality associated with BCWD in coho salmon. We have also demonstrated that use of ILM increases the efficacy of *F. psychrophilum* CSF259-93B.17 when used for immersion vaccination. Although adipose fin clipping may not be a feasible vaccination strategy in the hatchery setting, the current study suggests that physical abrasion prior to vaccination elicits greater protection particularly in fry that are too small to vaccinate by injection. This is especially true in the laboratory when an injection challenge protocol is required. While confirmation of these results under field conditions is needed, efficacy of this vaccine is enhanced when produced under iron-limited conditions and this may represent the most appropriate strategy for immersion vaccination of salmonid fry.

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